Thermodynamics of Inhibitor Binding to Mutant Forms of Glucoamylase from *Aspergillus niger* Determined by Isothermal Titration Calorimetry†

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ABSTRACT: We have investigated the binding of mutant forms of glucoamylase from *Aspergillus niger* to the inhibitors 1-deoxynojirimycin and acarbose. The mutants studied comprise a group of single amino acid replacements in conserved regions near the active site of the enzyme. For each mutant we have determined both the affinities for the two inhibitors and the thermodynamic state functions for binding using titration microcalorimetry. We find that acarbose binds to all the mutants with a wide range of binding constants ($10^4 < K_a < 10^{13}$ M^{-1}). In contrast, 1-deoxynojirimycin shows either binding at near wild-type affinity ($K_a \cong 10^4$ M^{-1}) or no detectable binding. The changes in the affinities of the mutant enzymes are rationalized in terms of the known three-dimensional structure of the wild-type enzyme with subsites 1, 2, and 3 being important for acarbose binding while only subsite 1 is critical for 1-deoxynojirimycin binding. In most of the mutants studied the magnitudes of the enthalpies and the entropies of binding of the mutant enzymes differed from those of the wild-type enzyme with the mutant enzymes having a relatively large portion of their binding energy composed of enthalpy and a relatively small proportion composed of entropy. The pattern of changes in the enthalpy and entropy is hypothesized to be due to changes in the structural complementarity of the binding pocket and the inhibitor.

The need for an improved understanding of the detailed molecular nature of the binding forces between proteins and their ligands is one of the great challenges currently facing protein biophysics. In order to make progress toward this goal, it will be necessary to obtain thermodynamic data on the binding reactions between proteins and their ligands; such data should include information on the enthalpies and entropies of binding as well as the free energies (Sturtevant, 1994). In particular, studies on mutant forms of a given enzyme have great potential to help decipher the relationship between the structure of the binding site and the nature of the resulting binding forces. The enzyme glucoamylase from *Aspergillus niger* (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) is an excellent model system for such studies due to both the extensive available knowledge of its structure and function and the fact that a large number of mutants have been constructed (Sierks et al., 1989, 1990, 1993; Sierks & Svensson, 1992, 1993, 1994; Frandsen et al., 1994; Svensson et al., 1994).

Glucoamylase catalyzes the hydrolysis of starch by removing a glucose residue from the nonreducing end of the sugar chain and is capable of hydrolyzing both α-(1→4)- and α-(1→6) linkages (Hiromi et al., 1966). *A. niger* produces glucoamylase in two forms known as G1 and G2.[1] The G1 form is a multidomain enzyme containing both a catalytic domain and a granular starch binding domain (SBD) which enables this form to hydrolyze raw starch; the G2 form contains only the catalytic domain (Svensson et al., 1982, 1983, 1986). Both forms of the enzyme have similar catalytic properties toward soluble substrates (Svensson et al., 1982). Like many other glycosylases, glucoamylase is inhibited by sugar analogues with a basic nitrogen atom adjacent to C-1 [for a review, see Bock and Sigurskjold (1990), Svensson and Sierks (1992) and Sigurskjold et al. (1994a)]. Two examples of this type of inhibitor are 1-deoxynojirimycin and the very strong inhibitor acarbose (Truscheit et al., 1981). Acarbose is a pseudotetrasaccharide analog whose nonreducing end contains a pseudosugar moiety with a double bond and a nitrogen atom instead of the glycosidic oxygen (Figure 1). 1-Deoxynojirimycin is a glucose analog with a nitrogen replacing the endocyclic oxygen and is deoxygenated at the anomeric center.

Crystal structures of the closely related glucoamylase from *Aspergillus awamori* var. *X100* uncomplexed (Aleshin et al., 1992, 1994b) and in complex with both 1-deoxynojirimycin (Harris et al., 1993) and acarbose (Aleshin et al., 1994a) have been published. Furthermore, a crystal structure of the complex between this glucoamylase and D-*gluco*-dihydroacarbose, a hydrogenated form of acarbose (Stoffer, et al., 1995), has become available very recently. These structures reveal that the catalytic domain of glucoamylase has an $(\alpha/\alpha)_6$-barrel fold with the active site cleft formed by the connecting segments between the N-termini of the inner helices and the C-termini of the preceding outer helices.

Because glucoamylases are used extensively in the starch industry for the production of high glucose and fructose

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[1] Abbreviations: SBD, starch binding domain; ITC, isothermal titration calorimetry; G1, full-length form of glucoamylase (amino acid residues 1−616); G2, truncated form of glucoamylase (amino acid residues 1−512) consisting of the entire catalytic domain as well as the linker region, but without the SBD.

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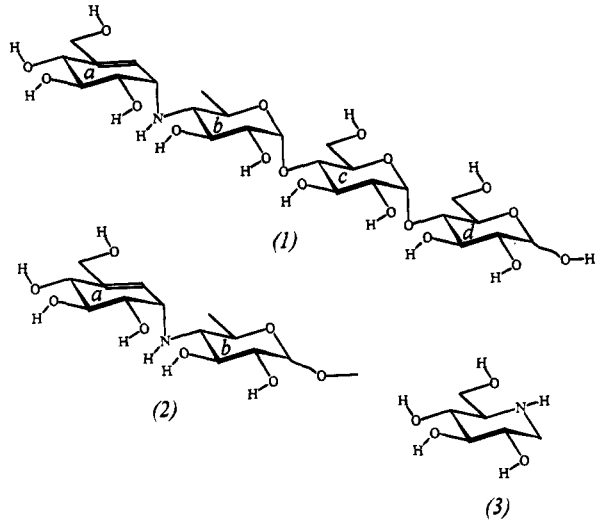

FIGURE 1: Structures of the inhibitors acarbose (1), *O*-methyl acarviosinide (2), and 1-deoxynojirimycin (3).

syrups as well as in brewing (Saha & Zeikus, 1989), this enzyme has been the focus of protein engineering efforts directed at further improving its industrial suitability. In the course of these studies a large number of mutants have been constructed by this and other laboratories. In particular it was found by aligning the structures of several closely related glucoamylases that these enzymes have six highly conserved regions (Itoh et al., 1987; Stoffer, 1994). A series of single amino acid substitutions has been made in five of these conserved regions and includes residues suggested to be of functional significance by the X-ray crystal structure. The resulting mutants have been characterized for enzymatic properties and temperature stability (see references in the Materials and Methods).

In addition to the availability of good structural information and a large number of available mutants, glucoamylase is an attractive target for a study of the effect of mutations on the thermodynamics of ligand binding since a similar study has already been made for the wild-type enzyme (Sigurskjold et al., 1994a). In this study isothermal titration calorimetry (ITC) (Wiseman et al., 1989; Bundle & Sigurskjold, 1994) was used to characterize the binding of wild-type glucoamylase G1 and G2 to several sugar-analog inhibitors including 1-deoxynojirimycin and acarbose. Both G1 and G2 bind specifically to these inhibitors, showing a very high affinity for acarbose ($K_a \cong 10^{12}$ M^{-1}) and a much weaker binding of 1-deoxynojirimycin ($K_a \cong 10^4$ M^{-1}). For both forms of the enzyme, binding to each of these inhibitors is accompanied by favorable changes in both the entropy and the enthalpy. The binding affinities of G1 and G2 are the same for a given ligand, but small compensatory changes in the entropy and enthalpy of binding are seen between the two forms. In a separate study we have used ITC to study the binding of inhibitors to an isolated fragment of glucoamylase containing mainly the SBD (Sigurskjold et al., 1994b). SBD binds both linear and cyclic polysaccharides with an enthalpic driving force and unfavorable entropy changes. The thermodynamic parameters of SBD are affected by the presence or absence of the catalytic domain.

In this paper we report microcalorimetric titration studies of the interaction between several mutant glucoamylases and the inhibitors 1-deoxynojirimycin and acarbose; these data on the thermodynamic effects of varying the protein are a natural complement to the previous data on the effects of varying the ligand. Many of the mutants studied continued to bind 1-deoxynojirimycin specifically, and all of them bound acarbose. The mutant enzymes exhibited both changes in their affinities for sugar analogs and changes in the magnitudes of the enthalpy and the entropy of binding these analogs. We discuss the implications of these changes in terms of the known structure of enzyme−inhibitor complexes and in terms of the important forces in ligand binding.

MATERIALS AND METHODS

Proteins and Inhibitors. Mutant forms of glucoamylase were constructed and purified essentially as described earlier. Details on the specific mutants and their enzyme kinetic properties are reported elsewhere: Trp120→Phe (Sierks et al., 1989); Asp176→Asn and Glu180→Gln (Sierks et al., 1990); Tyr48→Trp (Frandsen et al., 1994); Ser119→Tyr (Sierks & Svensson, 1994); Asn171→Ser, Gln172→Asn, Thr173→Gly, Gly174→Cys, and Tyr175→Phe (B. Stoffer, C. Dupont, T. P. Frandsen, J. Lehmbeck, and B. Svensson, unpublished); Ser185→His (B. Stoffer, J. Lehmbeck, and B. Svensson, unpublished results); Arg54→Lys, Arg54→Leu, Arg305→Lys, Asp309→Glu, and Trp317→Phe (Frandsen et al., 1995); Tyr50→Phe (Frandsen, 1993); Trp52→Phe (T. Christensen, unpublished). Most of the measurements were performed using the G1 form of glucoamylase, but for mutants Tyr48→Trp and Tyr175→Phe the G1 form was not available. In these cases the catalytic domain, prepared with subtilisin Novo (Stoffer et al., 1993), and the G2 form (Svensson et al., 1982) were used, respectively. Immediately before use the protein was dialyzed into 0.05 M sodium acetate buffer, pH 4.5. If necessary, solutions were spun for several minutes in a bench-top centrifuge to remove any visible precipitate. Concentrations of glucoamylase G1 and G2 were estimated spectrophotometrically at 280 nm using $\epsilon = 1.37 \times 10^5$ M^{-1} cm^{-1} and 1.09×10^5 M^{-1} cm^{-1}, respectively (Clarke & Svensson, 1984). Accurate estimates of the concentration of mutant protein were not necessary since the precise values could be found from the shape of the ITC binding isotherm (see below). Therefore, no corrections were made for changes in the extinction coefficient due to amino acid replacements which could be appropriate when tyrosine or tryptophan residues are involved. However, even for the mutants involving Tyr or Trp, good agreement between absorbance and calorimetric estimates was observed. In previous work (Sigurskjold et al., 1994a) it was established that excellent agreement exists between protein concentrations of wild-type glucoamylase measured spectrophotometrically and by ITC. Weighed amounts of the freeze-dried inhibitors 1-deoxynojirimycin, methyl α,β-acarviosinide, and acarbose were dissolved in buffer identical to that of the protein. No impurities were detectable in 500 MHz ^{1}H NMR spectra. Acarbose and 1-deoxynojirimycin were generous gifts from E. Möller, and methyl α,β-acarviosinide was prepared by F. Heiker, both at Bayer AG (Wuppertal, Germany).

Titration Calorimetry. Calorimetric measurements were carried out using an OMEGA titration microcalorimeter (MicroCal, Inc., Northampton, MA). This instrument has been described in detail by Wiseman et al. (1989). The

reference cell was filled with water, and the instrument was calibrated using standard electrical pulses. All solutions were thoroughly degassed by stirring under vacuum before use. Solutions of mutant glucoamylase were titrated with 20 identical injections of the inhibitor. The injection syringe, on which a stirrer paddle is mounted, stirred the solutions at 400 rpm ensuring immediate mixing. The enzyme concentration was between 0.05 and 0.1 mM. The concentration of the ligand solution was chosen to ensure that the protein would be close to saturation with ligand well before the final injection. In this way both heats of binding and heats of dilution could be measured in a single experiment. All experiments were performed at 27.0 ± 0.2 °C. Determination of the heat capacity would require several measurements at different temperatures. Unfortunately, limited amounts of the mutants precluded this. The peaks of the obtained thermograms were integrated using the ORIGIN software (MicroCal, Inc.) supplied with the instrument. A nonlinear regression fit to the isotherm was performed using either a previously described fitting program (Sigurskjold et al., 1991) or the ORIGIN data analysis routine. The fitting procedure gives the binding constant of the ligand, K_a, the heat of binding, $\Delta H°$, the concentration of binding sites (stoichiometry), and the heat of dilution of the ligand. From these quantities it is possible to calculate the free energy and the entropy of binding using standard thermodynamic relationships: $\Delta G° = -RT \ln K_a = \Delta H° - T\Delta S°$. Each titration was only carried out once, and the listed uncertainties represent three standard deviations (3σ confidence level) obtained from the nonlinear regression analysis (Bundle & Sigurskjold, 1994).

Displacement Experiments. For some of the mutants, the binding of acarbose was too tight to be measured by direct titration. The binding constant can be obtained from the titration isotherm only if the product of the binding constant and the protein concentration, K_a[protein], lies in the interval from 1 to 1000, and preferably between 10 and 100 (Wiseman et al., 1989). For those mutants with $K_a > 10^7$ M^{-1}, the protein concentrations which fall within this window are so low that the heat evolved during the reaction is below the detection limit. We have previously shown that the technique of displacement titration can be successfully used to measure the thermodynamics of binding in such cases, in particular for the binding of acarbose to wild-type glucoamylase (Sigurskjold et al., 1994a). Briefly, the solution of mutant glucoamylase is first titrated with a relatively weak inhibitor, either 1-deoxynojirimycin ($K_a \cong 10^4$ M^{-1}) or methyl α,β-acarviosinide ($K_a \cong 10^7$ M^{-1}), whose binding can be measured directly. The solution of protein plus inhibitor is then titrated with acarbose; the competition between the first inhibitor and the acarbose for the protein binding site reduces the apparent strength of the acarbose binding, and the titration curve can be fitted to give apparent values of K_{app} and ΔH_{app}. If there is a difference of several orders of magnitude in the binding constants of the first and second inhibitors, then the apparent values are simply related to the true values for acarbose (K_{ac}, $\Delta H°_{ac}$) by the equations

$$\Delta H°_{ac} \cong \Delta H_{app} + \Delta H°_{in} \quad (1)$$

and

$$K_{ac} \cong K_{app} \times K_{in} \quad (2)$$

where $\Delta H°_{in}$ and K_{in} are the enthalpy and binding constant for the first inhibitor determined independently.

In this paper we have extended the displacement technique to detect the binding of very low affinity ligands. Some of the mutants bound 1-deoxynojirimycin too weakly to detect directly (K_a[protein] < 1). In these cases displacement experiments could be used to test for binding which is weaker than that observable by direct titration if enough enzyme was available: two experiments are required. First, the enzyme is titrated with an inhibitor which is several orders of magnitude stronger than the weak inhibitor one desires to measure but still weak enough that the binding constant can be determined by direct titration; for several of the mutants acarbose fulfills these criteria. Then, in a second, separate experiment a displacement titration was performed as described above with 1-deoxynojirimycin as the first ligand and acarbose as the second ligand. The binding constant and enthalpy could then be found using eqs 1 and 2, but in this case K_{ac} and $\Delta H°_{ac}$ are directly determined by the first titration and $\Delta H°_{in}$ and K_{in} are the unknown quantities.

RESULTS AND DISCUSSION

Acarbose Binding. The binding constants and energies for the binding of several glucoamylase mutants to acarbose are shown in Table 1. It has previously been shown that acarbose binds specifically to G1 both at the active site (Sigurskjold et al., 1994b) and at a second site on the SBD (Belshaw & Williamson, 1990): in the wild-type enzyme, the active site has much greater affinity, $K_a \cong 10^{12}$ M^{-1}, than the SBD site, $K_a \cong 10^3$ M^{-1} (Sigurskjold et al., 1994b). In most cases only the strong binding to the active site is detected by ITC. However, in some cases, especially those experiments where a high concentration of acarbose was used, some evidence of binding to a second weaker site after saturation of the first site was detected in the shape of the isotherm: Figure 2 shows a typical thermogram and isotherm where such binding occurs. In this figure the first, sharp rise in the isotherm represents saturation of the first binding site, and the second, slow almost linear rise represents weak binding to the second site. The nearly linear shape of the second transition is typical of weak binding for which saturation is far from approached within the concentration range of the ligand. In cases where such isotherms were observed, the thermodynamic constants for each of the two binding sites could be obtained by fitting the isotherm with the two-independent-site binding model of the ORIGIN software package. In all cases the thermodynamic parameters for the second binding site agreed with those previously determined for the binding between acarbose and the isolated (wild-type) SBD (Sigurskjold et al., 1994b). We do not understand the reason why this binding is observed in only some cases and not others. Because all of the mutant enzymes studied had the mutation in the catalytic domain, only the data for the stronger binding to the active site are given in Table 1; mutants where SBD binding was seen are indicated with a superscript "f". Because the two-site model has both a larger number of parameters and a less accurate correction for the heat of dilution than the one-site model, the uncertainties in $\Delta H°$and $T\Delta S°$ may be slightly underestimated for those mutants where this model was used. In the case of weakest binding, Arg305→Lys, it is possible that the active site and SBD binding data are confounded.

Table 1: Association Constants and Thermodynamic Parameters for the Binding of Acarbose to the Active Site of Wild-Type and Mutant Glucoamylase G1 Measured at pH 4.5, 27 °C

enzyme	K_a (M^{-1})	$\Delta H°$ (kJ mol^{-1})	$-T\Delta S°$ (kJ mol^{-1})	$\Delta G°$ (kJ mol^{-1})
wild type, G1	$(9.4 \pm 6.0) \times 10^{11}$	-32.8 ± 1.4	-36.1 ± 2.1	-68.9 ± 1.6
wild type, G2	$(8.8 \pm 3.2) \times 10^{11}$	-40.6 ± 0.1	-27.6 ± 0.9	-68.2 ± 0.9
Tyr48→Trp[a,b]	$(2.0 \pm 1.7) \times 10^{5}$	-10.9 ± 3.7	-19.6 ± 4.3	-30.5 ± 2.1
Tyr50→Phe[c,f]	$(1.6 \pm 1.0) \times 10^{12}$	-39.6 ± 1.8	-30.6 ± 2.4	-70.2 ± 1.6
Trp52→Phe[c,f]	$(2.4 \pm 0.9) \times 10^{11}$	-53.2 ± 0.9	-12.2 ± 2.9	-65.4 ± 2.8
Arg54→Lys	$(8.2 \pm 2.7) \times 10^{6}$	-41.2 ± 0.4	1.5 ± 0.9	-39.7 ± 0.8
Arg54→Leu	$(3.9 \pm 1.5) \times 10^{6}$	-36.0 ± 0.8	-1.8 ± 1.3	-37.8 ± 1.0
Ser119→Tyr[c]	$(9.3 \pm 7.2) \times 10^{11}$	-29.9 ± 1.5	-38.9 ± 2.5	-68.8 ± 2.0
Trp120→Phe	$(1.0 \pm 0.3) \times 10^{7}$	-29.0 ± 0.2	-11.3 ± 0.7	-40.3 ± 0.6
Asn171→Ser[c,f]	$(1.4 \pm 0.3) \times 10^{12}$	-51.5 ± 1.5	-18.2 ± 1.6	-69.7 ± 0.6
Gln172→Asn[c,f]	$(2.5 \pm 2.5) \times 10^{12}$	-47.1 ± 1.1	-24.2 ± 2.0	-71.3 ± 1.7
Thr173→Gly[c,f]	$(1.6 \pm 0.2) \times 10^{12}$	-53.5 ± 0.6	-16.6 ± 0.7	-70.1 ± 0.3
Gly174→Cys[c]	$(3.5 \pm 9.0) \times 10^{12}$	-35.1 ± 3.3	-36.9 ± 7.2	-72.0 ± 6.3
Tyr175→Phe[d,e]	$(1.4 \pm 0.9) \times 10^{13}$	-37.2 ± 1.8	-38.3 ± 2.4	-75.4 ± 1.6
Asp176→Asn[c]	$(1.7 \pm 1.5) \times 10^{11}$	-39.5 ± 1.1	-25.0 ± 2.5	-64.5 ± 2.2
Glu180→Gln	$(2.8 \pm 1.4) \times 10^{4}$	-19.8 ± 2.9	-5.7 ± 3.2	-25.5 ± 1.3
Ser185→His[c]	$(1.2 \pm 0.8) \times 10^{11}$	-54.3 ± 1.0	-9.3 ± 1.1	-63.7 ± 0.5
Arg305→Lys	$(9.3 \pm 6.4) \times 10^{4}$	-30.4 ± 6.2	1.9 ± 6.4	-28.5 ± 1.7
Asp309→Glu[c]	$(2.0 \pm 0.7) \times 10^{10}$	-31.1 ± 1.2	-28.1 ± 1.6	-59.2 ± 0.9
Trp317→Phe	$(8.6 \pm 7.8) \times 10^{7}$	-35.4 ± 1.4	-10.2 ± 2.6	-45.6 ± 2.3

[a] Values were measured in a displacement experiment where no binding of 1-deoxynojirimycin was detected. However, it is possible that binding occurred below the detectable limit. Therefore, the uncertainties may be larger than indicated. [b] Catalytic domain, prepared with subtilisin Novo (Stoffer et al., 1993). [c] Displacement experiment, 1-deoxynojirimycin. [d] Displacement experiment, methyl α,β-acarviosinide. [e] G2 form. [f] Isotherm fit with two-site binding model, see text for explanation.

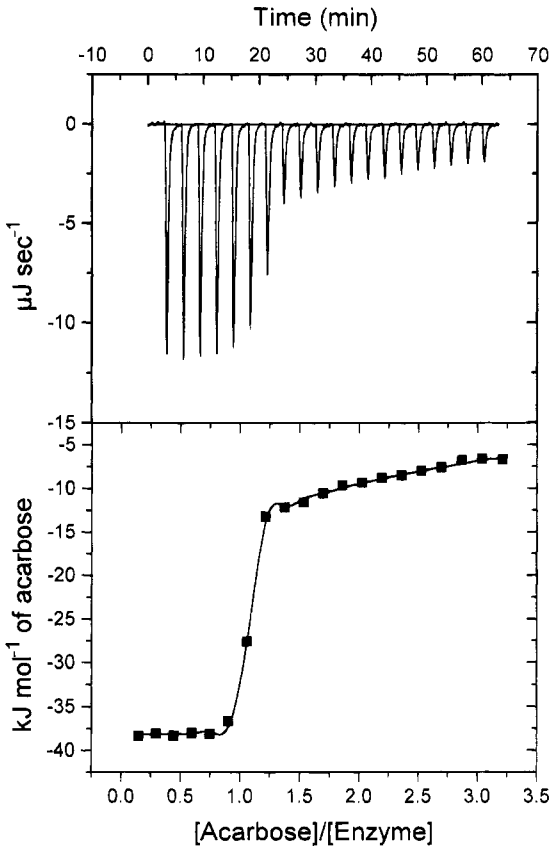

FIGURE 2: Thermogram (top) and isotherm (bottom) for the titration of N171S glucoamylase with acarbose in which binding to a second site is observable.

However, the observed binding cannot be due to the SBD alone since in both cases the measured enthalpy is more favorable than that expected for the SBD (Sigurskjold et al., 1994b). Therefore, while the thermodynamic values for this mutant may not be quantitatively correct, it is clear that it gives accurate, qualitative information on the nature of the binding. Thus, acarbose binds to the active site of Arg305→Lys with weak affinity and close to wild-type enthalpy.

The mutant proteins tested all bound acarbose, but with a very wide range of binding constants: from approximately 10^3 to 10^{13} M^{-1}. This variation in affinity for acarbose can be rationalized using available knowledge on the three-dimensional structure of the enzyme. Figure 3 shows a view of the fold of glucoamylase with the location of mutated residues marked by a plus sign. The active site of glucoamylase is a funnel shape formed by residues from segments from the N-termini of the inner α-helices to the C-termini of the outer helices (Aleshin et al., 1992). This pocket is hypothesized to contain seven consecutive subsites, each of which binds one glucose unit in an extended substrate with cleavage occurring between subsites 1 and 2 (Hiromi et al., 1983). A closer view of the active site showing only the catalytic carboxyl residues, Glu179 and Glu400, and residues mentioned in the text is shown in Figure 4. The crystal structures of the protein in complex with acarbose (Aleshin et al., 1994a), D-*gluco*-dihydroacarbose (Stoffer et al., 1995), and 1-deoxynojirimycin (Harris et al., 1993) suggest that both hydrogen bonding to sugar OH groups and stacking of sugar rings against aromatic groups are important for positioning and binding substrates and inhibitors. Those mutants with the greatest reduction in affinity for acarbose can be characterized as belonging to two categories: (1) mutations in groups directly involved either in hydrogen bonds with the substrate or in stacking against sugar, and (2) mutations in residues which stabilize one of the important sugar binding or catalytic residues.

The first category includes the mutants Arg54→Lys, Arg54→Leu, Trp120→Phe, Glu180→Gln, and Arg305→Lys. The crystal structure of the acarbose complex (Aleshin et al., 1994a) reveals that Arg54 hydrogen bonds to the critical OH-4 group in the first sugar ring. Similarly, Glu180 hydrogen bonds to the OH-2 group in the second ring. Arg305 hydrogen bonds with both the OH-2 group in the

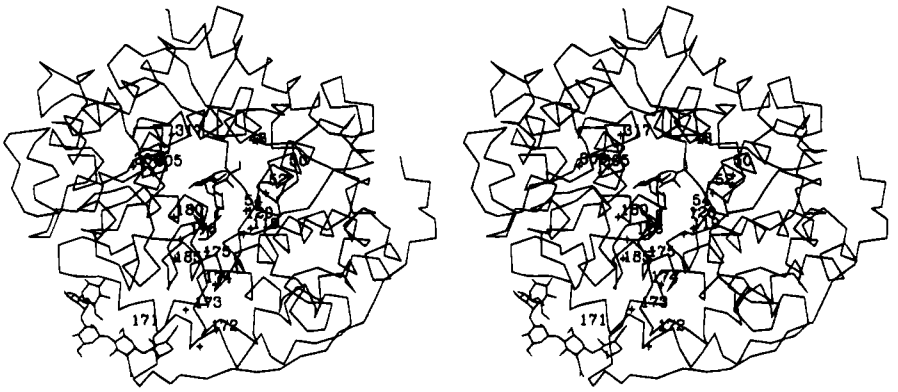

FIGURE 3: Stereoview of the fold of glucoamylase from *A. awamori var. X100* in complex with acarbose (Aleshin et al., 1994a). Amino acids discussed in this work are indicated by numbers and by the symbol (+).

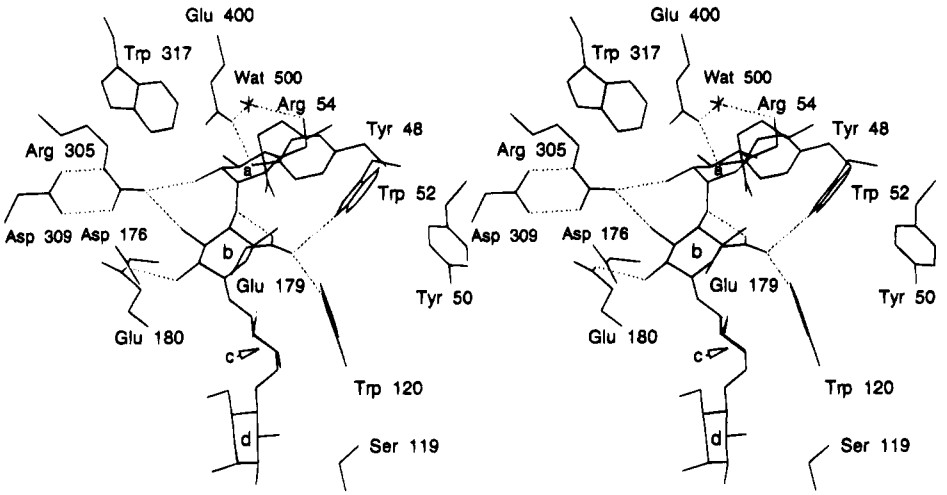

FIGURE 4: Stereoview of the active site of glucoamylase from *A. awamori var. X100* in complex with acarbose (Aleshin et al., 1994a). Only the catalytic carboxyl groups and amino acids discussed in the text have been included. Hydrogen bonds are represented by dashed lines.

first and the OH-3 group in the second ring. Trp120 is both directly involved in binding by stacking against the third sugar ring and in stabilizing a catalytically important residue by hydrogen bonding to the catalytic acid Glu179; this residue is important for both catalysis and for stabilizing a substrate intermediate complex (Sierks et al., 1989; Olsen et al., 1993). Since acarbose is supposed to act as a transition state mimic (Truscheit et al., 1981; Sigurskjold et al., 1994a), loss of intermediate stabilization should translate directly to lower affinity for this inhibitor.

The second group with reduced acarbose affinity includes the mutants Tyr48→Trp, Trp52→Phe, Asp309→Glu, and Trp317→Phe. Tyr48 participates in a hydrogen bond network which stabilizes the catalytic base Glu400 (Harris et al., 1993; Frandsen et al., 1994). Tyr48 hydrogen bonds through Glu400 to the water molecule (Wat500) which presumably makes the nucleophilic attack on C-1 during glycoside hydrolysis. Mutation of this residue may cause disruption of important interactions between the inhibitor and the catalytic groups in subsite 1 and 2. Trp52 forms part of a hydrophobic barrel which stabilizes Trp120 (Harris et al., 1993; Svensson et al., 1994). Mutation to phenylalanine causes only a mild reduction in affinity for acarbose. Asp309 is involved in a hydrogen bond network with the critically important Arg305. However, Asp309→Glu has only a mild reduction in acarbose affinity. Finally, Trp317 stabilizes Arg305 and the catalytic base Glu400 through hydrophobic interactions (Frandsen et al., 1995). All other amino acid substitutions studied (50, 119, 171−175, 176, 185) had little or no affect on the affinity of the enzyme for acarbose. The substitution of Phe for Tyr175 actually resulted in a small increase in the affinity of the enzyme for acarbose. We conclude from this that if the major hydrogen bond and stacking interactions between acarbose and G1 remain, other changes in or near the binding pocket of the enzyme can be accommodated without substantial loss of affinity for the inhibitor.

Unlike the binding constant and the free energy, which remain of wild-type magnitude for many of the mutants, the binding entropies and enthalpies show consistent changes from the wild-type for nearly all of the mutant enzymes studied (Table 1). Thus for those mutants with nearly wild-type affinity for acarbose, a greater proportion of the binding energy is contributed by the enthalpy of binding with a compensating reduction in the favorable entropy of binding compared to the wild-type G1 form. This pattern is observed for all of the mutants (albeit weakly for some) except for the Ser119→Tyr and Gly174→Cys mutants which have wild-type entropy and enthalpy. The increase in the proportion of the binding energy provided by the enthalpy of binding is even more pronounced in those mutants which have reduced affinity for acarbose. In some of these cases, the contribution of the entropy of binding actually becomes unfavorable but is partially compensated by very large heats of association.

Table 2: Association Constants and Thermodynamic Parameters for the Binding of 1-Deoxynojirimycin to the Active Site of Wild-Type and Mutant Glucoamylase G1 Measured at pH 4.5, 27 °C

enzyme	K_a (M^{-1})	$\Delta H°$ (kJ mol^{-1})	$-T\Delta S°$ (kJ mol^{-1})	$\Delta G°$ (kJ mol^{-1})
wild type, G1	$(4.7 \pm 1.7) \times 10^4$	-7.0 ± 0.9	-19.9 ± 1.3	-26.9 ± 0.9
wild type, G2	$(3.3 \pm 0.2) \times 10^4$	-11.2 ± 0.1	-14.5 ± 0.2	-25.8 ± 0.1
Tyr48→Trp[a]	$<10^4$			
Tyr50→Phe	$(3.5 \pm 1.8) \times 10^4$	-10.0 ± 1.8	-16.1 ± 2.2	-26.1 ± 1.3
Trp52→Phe	$(5.0 \pm 0.5) \times 10^3$	-18.8 ± 0.8	-2.4 ± 0.9	-21.2 ± 0.2
Arg54→Leu[c]	<10			
Ser119→Tyr	$(7.2 \pm 3.3) \times 10^4$	-9.0 ± 1.5	-19.0 ± 1.9	-27.9 ± 1.1
Trp120→Phe	$(3.2 \pm 0.4) \times 10^4$	-28.9 ± 1.8	3.1 ± 1.9	-25.9 ± 0.3
Asn171→Ser	$(5.5 \pm 1.9) \times 10^4$	-15.2 ± 1.4	-12.0 ± 1.7	-27.2 ± 0.9
Gln172→Asn	$(2.9 \pm 0.2) \times 10^4$	-13.8 ± 0.2	-11.8 ± 0.3	-25.6 ± 0.2
Thr173→Gly	$(5.5 \pm 0.7) \times 10^4$	-17.3 ± 0.6	-9.9 ± 0.7	-27.2 ± 0.3
Gly174→Cys	$(4.6 \pm 4.5) \times 10^4$	-9.9 ± 3.3	-16.8 ± 4.1	-26.8 ± 2.4
Tyr175→Phe[b]	$(7.8 \pm 3.9) \times 10^3$	-12.0 ± 5.1	-10.3 ± 5.3	-22.3 ± 1.3
Asp176→Asn	$(2.7 \pm 0.1) \times 10^4$	-13.5 ± 0.1	-11.9 ± 0.2	-25.4 ± 0.1
Ser185→His	$(4.0 \pm 0.1) \times 10^3$	-22.4 ± 0.8	1.7 ± 0.8	-20.7 ± 0.1
Glu180→Gln	$(1.6 \pm 0.2) \times 10^4$	-14.2 ± 1.1	-10.0 ± 1.2	-24.2 ± 0.3
Asp309→Glu	$(5.1 \pm 1.1) \times 10^4$	-11.7 ± 0.9	-15.4 ± 1.1	-27.1 ± 0.5

[a] Catalytic domain, prepared with subtilisin Novo (Stoffer et al., 1993). [b] G2 form. [c] Displacement experiment, see Materials and Methods.

The magnitude of $\Delta\Delta G°$, $\Delta G°_{WT} - \Delta G°_{Mut}$, and the fraction of these changes due to the contributions of $\Delta\Delta H°$ and $\Delta\Delta S°$ can be used to illustrate both the difficulty involved in attributing the thermodynamic changes to a structural cause and the importance of obtaining $\Delta H°$ and $\Delta S°$ as well as $\Delta G°$. For example, on the basis of the wild-type crystal structure, mutants Arg54→Lys and Arg305→Lys, are both expected to lose one hydrogen bond to the substrate; the loss of this hydrogen bond might naively be expected to be the dominant factor in any changes to the thermodynamics of binding. However, upon examining the data, we see that $\Delta\Delta G°$ is quite different for the two mutants, approximately -29 kJ mol^{-1} for Arg54→Lys and -40 kJ mol^{-1} for Arg305→Lys; moreover, the components of the change due to enthalpy and entropy are different in the two cases. For Arg54→Lys the less favorable $\Delta G°$ is entirely due to a large loss of favorable entropy, the enthalpy is actually more favorable than the wild-type while for Arg305→Lys, $\Delta\Delta G°$ is due to unfavorable changes in the entropy while the enthalpy is of approximately the same magnitude as for the wild type. A loss of a hydrogen bond is usually expected to cause unfavorable changes in enthalpy and favorable changes in entropy (Hawkes et al., 1984; Shortle et al., 1988).

Although it is not possible to assign a given change in the thermodynamic parameters to a definite structural cause, the observed pattern of increases in the heats of association and corresponding decreases in the favorable entropy seen for many of the mutants confirms that most of the mutant enzymes bind acarbose in a slightly different fashion than the wild-type enzyme. The likely sources of increased enthalpy include more or stronger hydrogen bonds and/or increased van der Waals contact between surfaces; increases in hydrogen bonding or van der Waals interactions will also lead to a reduction in $\Delta S°$. The loss of favorable entropy could also be due to other causes. In many of the mutants the loss of favorable entropy is probably partially due to a reduction in the amount of hydrophobic surface buried in the interaction as the hydrophobic effect is the largest source of favorable entropy available in association reactions (Finkelstein & Janin, 1989; Spolar & Record, 1994). This suggests that in the wild-type complex burial of hydrophobic surfaces does indeed take place. A reduction in the number of displaced water molecules during binding need not be incompatible with increased hydrogen bonds and van der Waals contacts since both of these types of interactions can be water mediated.

The changes in the binding energetics observed for many of the mutants studied are suggestive of small but long-ranging changes in the shape or flexibility of the binding pockets of these enzymes compared to the wild-type enzyme. These changes could affect the orientation of the hydrogen-bonding residues and the shape complementarity between the pocket and the inhibitor. An indication of how far ranging these small changes may be is given by the case of Tyr175→Phe, a mutation located near subsites 4−6 (B. Stoffer, C. Dupont, T. P. Frandsen, J. Lehmbeck, and B. Svensson, unpublished results) which are far from the catalytic center and are usually thought to have very minor binding energies (Hiromi et al., 1983; Ermer et al., 1993). Nevertheless, since this mutant has 10-fold increased affinity for acarbose over the wild-type, this conservative change has significantly affected the fashion in which the enzyme binds the inhibitor. Similarly, mutations in residues 171−173, which are far from the catalytic center (see Figure 3), cause a decrease of 14−21 kJ mol^{-1} in enthalpy of binding (more favorable binding) with compensating unfavorable changes in the entropy, suggesting that a change in this region leads to less water displacement during complex formation than for the wild-type enzyme. Mutation of Asn171 or Thr173 induces a loss of the *N*-linked carbohydrate of Asn171 which may alter the shape of the binding pocket.

Interestingly, the trend toward higher enthalpies of association for the mutants than for the wild-type G1 form is also seen when wild-type G1 and G2 are compared. The mere absence of the SBD is thus significant, suggesting that the shape of the binding pocket of glucoamylase is influenced by the presence or absence of SBD and these changes shift the balance between enthalpy and entropy in favor of enthalpy without affecting the affinity.

1-Deoxynojirimycin Binding. Since, for the wild-type enzyme, 1-deoxynojirimycin binding is considerably weaker ($K_a \cong 10^4$ M^{-1}) than for acarbose, only those mutants with reasonable residual activity were tested for 1-deoxynojirimycin binding. The binding constants and energies for these mutants are shown in Table 2. The pattern for 1-deoxynojirimycin binding is rather different from the acarbose

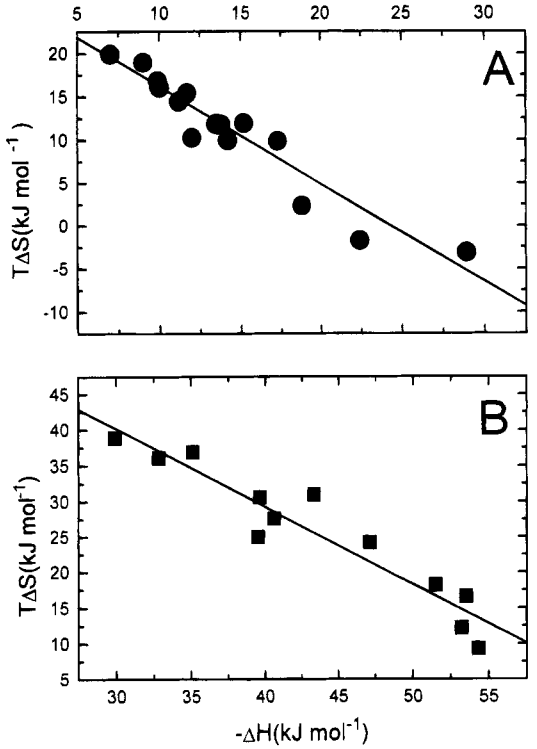

FIGURE 5: Entropy−enthalpy compensation plots for the binding of mutant glucoamylase G1 with 1-deoxynojirimycin (A) and acarbose (B). Note the different axes scales in panels A and B.

pattern. For those mutants tested this inhibitor either bound with near wild-type affinity or else showed no detectable binding. At the 1-deoxynojirimycin concentrations used the detection limit would be $\leq 10^2$ M^{-1}. For the mutant Arg54→Leu, we were able to verify that the affinity for 1-deoxynojirimycin was less than 10^1 M^{-1} by using displacement experiments. 1-Deoxynojirimycin is a monosaccharide analog with its main binding site in subsite 1 and a second binding site with much weaker affinity in subsite 2 (Harris et al., 1993). Binding to the second site is not detectable with the sensitivity of our instrument; therefore, all data reported in Table 2 apply to the binding in subsite 1. The data suggest that if mutations are made in or near subsite 1, as in the mutants Tyr48→Trp and Arg54→Leu, 1-deoxynojirimycin binding is abolished. However, as with the binding of acarbose, the conservative substitution Tyr50→Phe is tolerated. Mutations outside subsite 1 appear to cause at most small reductions in affinity. A striking example is the near wild-type affinity for 1-deoxynojirimycin of the Glu180→Gln mutant that has dramatically reduced affinity for acarbose. Residue 180 is located in subsite 2 and is important for both catalysis and ground-state binding (Sierks et al., 1990; Sierks & Svensson, 1992). Interestingly, the mutant Tyr175→Phe has one of the smallest while still measurable affinities for 1-deoxynojirimycin in spite of its enhanced affinity for acarbose. Apparently the optimal binding pocket is different for the two inhibitors.

All of the mutants show increased enthalpies of reaction with reductions in the favorable entropy of binding for 1-deoxynojirimycin. Again in some cases the entropy of binding is actually unfavorable. However, 1-deoxynojirimycin binding differs from acarbose binding in that it shows strong entropy−enthalpy compensation. Figure 5A shows an entropy−enthalpy compensation plot for 1-deoxynojirimycin. This strong correlation between entropy and enthalpy implies that mutations in the enzyme, while changing the binding interactions, do not greatly change the affinity. In the mutants which still bind 1-deoxynojirimycin, only relatively weak, less specific interactions are affected. In fact, a similar plot can be constructed for acarbose binding if those mutants in which specific interactions are changed (groups 1 and 2 described above) are excluded. Such a plot is shown in Figure 5B; in this plot only mutants with $K_a > 10^{11}$ M^{-1} are included. Such entropy−enthalpy compensation plots are usually interpreted as evidence for the involvement of water molecules in the reaction and have been observed for a wide variety of reactions in aqueous solution (Lumry & Rajender, 1970) including protein−sugar binding reactions (Hindsgaul et al., 1984; Brummell et al., 1993). In the case of sugar binding reactions, the most likely scenario for the involvement of water is displacement of water molecules which were previously bound to the protein by the incoming carbohydrate. This displacement of water molecules by a bound inhibitor has been observed by comparing the crystal structures of the native and complexed glucoamylase for both 1-deoxynojirimycin (Harris et al., 1993) and acarbose (Aleshin et al., 1994a,b).

The binding energies and enthalpies for 1-deoxynojirimycin also show evidence for small but long-range changes in the structure of the binding pocket upon mutation. This can be seen most clearly by examining the data for mutations far from the catalytic center such as, for example, Asn171→Ser, Gln172→Asn, and Tyr175→Phe. These mutations show large changes in the binding entropies and enthalpies compared to the wild-type. In the case of Tyr175→Phe there is also a statistically significant although small reduction in binding affinity. This evidence for changes in the binding pocket is a good example of the additional information provided by calorimetry over methods which determine only kinetic parameters. All three of these mutants have near wild-type catalytic parameters k_{cat} and K_M for long substrates as well as close to normal stability to heat and protease digestion (B. Stoffer, C. Dupont, T. P. Frandsen, J. Lehmbeck, and B. Svensson, unpublished).

Comparison with Kinetic Data. All of the mutants characterized here have been tested in separate studies to determine their kinetic parameters on small oligosaccharide substrates. Therefore we can look for correlations between the calorimetry data and the kinetic parameters to better understand the mechanism of inhibition of acarbose and 1-deoxynojirimycin. In order to perform this comparison, we have constructed free energy relations, (log-log) plots of K_a for each of the inhibitors vs k_{cat}, K_M, and (k_{cat}/K_M) values for hydrolysis of maltose and maltoheptaose. The plots were not significantly different for the two different substrates.

For acarbose the parameters which show a correlation with affinity are k_{cat} and (k_{cat}/K_M). The correlation between K_a and (k_{cat}/K_M) is stronger than that between k_{cat} and K_a. The plot of K_a vs (k_{cat}/K_M) for maltose is shown in Figure 6; the plots of K_a vs k_{cat} and K_M are not shown. The solid line represents a least-squares linear fit to all of the data. Although there is considerable scatter about this line, it is nevertheless apparent that a reasonably good correlation between the affinity for acarbose and the turnover number exists. The fact that K_a correlates with (k_{cat}/K_M) and k_{cat} but not with K_M indicates that the interactions between acarbose and the enzyme more closely resemble those of the transition-state complex than ground-state binding. However, it is clear

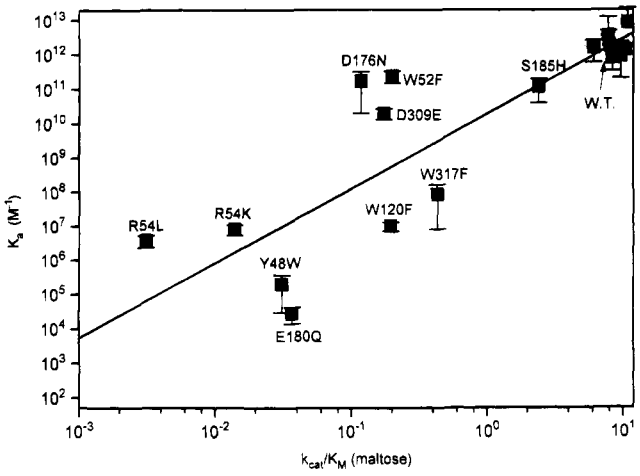

FIGURE 6: Correlation between the affinity (K_a) of mutant forms of glucoamylase G1 for acarbose and their catalytic efficiency (k_{cat}/K_M). The axes in this plot are on a logarithmic scale, and each point represents a mutant. The wild-type enzymes G1 and G2 are located in the upper right hand corner of the plot. The solid line is a least squares linear fit to the data, slope $\cong 2$, $r = 0.88$). Data for the kinetic parameters are from references listed in Materials and Methods.

from previous pre-steady-state kinetics measurements (Olsen et al., 1993) that acarbose is not a true transition-state analog. In fact, because of the double bond in the first ring, acarbose cannot assume the half-chair conformation presumed to occur in a true substrate during catalysis. Figure 6 indeed reinforces the unique nature of the interaction between acarbose and the enzyme since the slope of the best fit line is greater than 2, whereas for a true transition-state mimic the slope of the correlation line is expected to be unity (Bartlett & Marlowe, 1983).

The binding of acarbose to glucoamylase is remarkable both because it is neither ground-state nor transition-state like and because it is the strongest binding ever observed between a carbohydrate and a protein. The two most anomalous mutants, i.e., those farthest from the correlation line: Glu180→Gln and Asp176→Asn, may offer some insight into the special interactions between acarbose and glucoamylase. Both of these mutants have a greatly reduced (k_{cat}/K_M), 300 and 100 times smaller than the wild-type, respectively. Yet Asp176→Asn binds acarbose with a nearly normal K_a, only 4 times less than wild-type, while Glu180→Gln has K_a reduced by more than seven orders of magnitude. Both of these residues are located in or near subsite 2 (see Figure 4); residues in this subsite are assumed to be crucial both for inhibitor binding and for catalysis. Glu180 is directly hydrogen bonded to acarbose, and Asp176 is hydrogen bonded to the backbone carbonyl group of the catalytic acid Glu179 (Harris et al., 1993). In previous pre-steady-state kinetic measurements acarbose was shown to bind to glucoamylase in a three-step mechanism: a loosely associated enzyme inhibitor complex undergoes a slow conformational change to form a tightly bound complex which is presumably somewhat similar to the transition state of a true substrate; the tightly bound complex then undergoes a third reaction step which parallels the catalytic step in enzyme−substrate reactions (Olsen et al., 1993). Furthermore, residue 180 was shown to be important in the step leading to the tightly bound enzyme−inhibitor and enzyme−substrate complexes (Olsen, 1994). Probably the interactions between glucoamylase and acarbose involve similar residues in the first two reaction steps, but the third step is unique to acarbose. We propose that the catalytic residues play a much smaller role in this third step than they would in the hydrolysis of substrate. In this scenario, mutation of residue Glu180, which is important in the second step, would severely reduce the ability of acarbose to bind. However, mutations such as Asp176→Asn which perturb the catalytic residues do not greatly affect acarbose affinity. While resemblance between the tight enzyme−inhibitor complex and the transition state would account for the observed correlation between K_a and (k_{cat}/K_M), the fact that the catalytic residues are less important in the enzyme−acarbose complex than in the enzyme−substrate complex would account for the relatively weak correlation and the anomalous slope.

In contrast to the situation for acarbose, none of the free energy relations for 1-deoxynojirimycin showed any correlation between the kinetic parameters and affinity for the inhibitor (data not shown). This could have been expected since these mutants show a wide range of catalytic efficiency and very little variation in affinity for 1-deoxynojirimycin. 1-Deoxynojirimycin is usually thought of as a substrate analog; however, the lack of correlation between K_a and K_M must cast doubt on this view. It seems likely that the endocyclic nitrogen atom in 1-deoxynojirimycin elicits unique interactions between this inhibitor and the enzyme which are the major contributors to the binding energy as supported by the fact that this enzyme has virtually no affinity for glucose (Hiromi et al., 1982). Furthermore, subsite mapping suggests that subsite 2 has the strongest affinity for glucose (Hiromi et al., 1983; Ermer et al., 1993), yet 1-deoxynojirimycin binds preferentially in subsite 1 (Harris et al., 1993).

Conclusions. The strength of the binding between the inhibitors 1-deoxynojirimycin and acarbose and mutant forms of glucoamylase can be understood in terms of the known three-dimensional structure of the enzyme. In particular, subsite 1 is the most crucial for 1-deoxynojirimycin binding, and subsites 1, 2, and 3 are all important for acarbose binding. For most of the mutants, both inhibitors bind with favorable contributions from enthalpy and entropy suggesting that hydrophobic interactions are important driving forces in the binding. Many of the mutants bound inhibitors with more favorable enthalpy and less favorable entropy than the wild-type enzyme suggesting that single amino acid substitutions can cause small and also long-range changes in the shape of the binding pocket.

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